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SMAD signaling and airway cell fate determination

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Thesis

SMAD SIGNALING AND AIRWAY CELL FATE DETERMINATION

by

BRITTANY WILSON-MIFSUD

B.A., University of California, Santa Barbara 2017

Submitted in partial fulfillment of the
requirements for the degree of
Master of Science

2021

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SMAD SIGNALING AND AIRWAY CELL FATE DETERMINATION

BRITTANY WILSON-MIFSUD

ABSTRACT

Background: Goblet cell hyperplasia is a key feature of asthma and other airway diseases. It is likely regulated by the BMP/TGF- β /SMAD signaling pathway which is expressed in low levels in basal and goblet cells but highly active in other differentiated cell types. Therefore, SMAD signaling may be the main determinant of goblet vs non-mucus cell fate in the airway.

Objective: To determine the role of SMAD in goblet cell fate determination in the airway utilizing a knockout SMAD4 cell line.

Methods: Human adult and mouse tracheal SMAD4 knockout basal cell lines were generated. SMAD4 deletion was confirmed with western blot for both cell lines. Cells were cultured using an air-liquid interface to stimulate normal physiologic differentiation.

Results: Raised levels of goblet cells were noted in the human ALI. No ciliated cells were found in culture and club cell generation was also drastically lowered.

Conclusions: In human cells, differentiation of goblet cells was increased by SMAD deletion while differentiation of non-mucus cells was lowered. Lack of ciliated or normal levels of club cells after an extended culture suggests SMAD signaling is required for a non-mucus cell fate. These data support a role for SMAD signaling in both goblet cell generation and cell differentiation.

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LIST OF ABBREVIATIONS

| | |
|--------------------|---|
| AAV-Cre-GFP | Adeno-associated virus expressing Cre recombinase tagged with GFP |
| BMP | Bone morphogenic protein |
| Co-SMAD | “Co-factor” SMAD4 |
| DAPI | 4',6-diamidino-2-phenylindole (cell nucleus stain) |
| DMEM/F-12 | Dulbecco's Modified Eagle Medium/Modified Nutrient Mixture F-12 |
| FACS | Fluorescence-Activated Cell Sorting |
| FBS | Fetal Bovine Serum |
| GFP | Green Fluorescent Protein |
| IL-13 | Interleukin-13 |
| IRB | Institutional review board |
| PBS | Phosphate Buffered Saline |
| pHIV-H2B-eGFP | Plasmid expressing Human Histone H2B protein tagged with GFP |
| RPMI | RPMI 1640 medium |
| SMAD4 | Mothers against decapentaplegic homolog 4 |
| TGF / TGF- β | Transforming growth factor beta |

INTRODUCTION

Goblet cell hyperplasia or metaplasia leading to excessive mucus production is a common factor in most inflammatory airway diseases including cystic fibrosis, COPD, and asthma (Fig. 1). Hyperplasia of goblet cells driven by cytokine signaling is one of the key features of airway remodeling associated with asthma and is found in even mild cases that have few other clinical symptoms (Vieira et al., 2019). In addition to hyperplasia of standard goblet cells in the asthmatic lung, there is evidence of metaplasia including a muco-ciliary cell type that has features of both goblet and ciliated cells (Vieira et al., 2019). While there are a variety of secretory cells in the airway, the large population of goblet cells in disease states (Fahy et al., 2010) make them an important component of overall mucus production. Understanding the regulation of goblet cell differentiation through SMAD signaling can offer new targeted treatments for airway diseases.

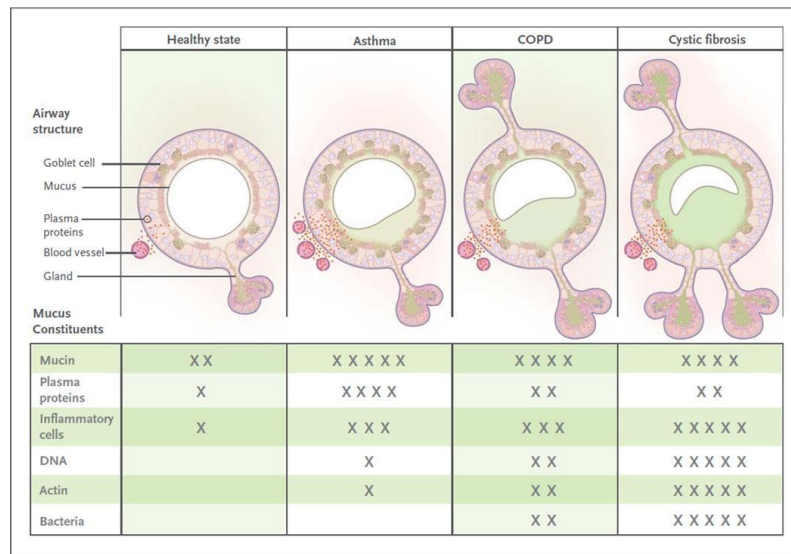


Figure 1. Examples of altered mucus production in different airway diseases (Fahy et al., 2010)

Lung epithelium and mucus production

There are different types of secretory cells in the different levels of the lung with goblet cells appearing in greatest numbers above the level of the bronchioles (McDowell et al., 1978). The major secretory cell below this level is the club cell (Widdicombe et al., 1982). In contrast to human lung epithelium which always has goblet cells present, mice require stimulus to produce goblet cells and have almost none under normal conditions (McCauley et al., 2015). Secretory cells are generally derived from the airway stem cell, the basal cell (Inayama et al., 1989). Goblet cell production from non-basal cell types has also been noted, mediated by inflammatory cytokine signaling including induction of the mucus-ciliary cell type and transdifferentiation (Tyner et al., 2006).

Normal mucus production in the lungs by all types of secretory cells creates a thin layer of mucus that traps small particles and pathogens that are inhaled into the airways. The beating of cilia on ciliated cells then moves the mucus out of the airways in mucociliary clearance. The constant clearance of mucus maintains the open airways and provides an important component of innate immunity by removing trapped pathogens and particles from the lungs (Wanner et al., 1996). Defensive functional molecules such as lysozyme, an anti-microbial, are also secreted and incorporated into the mucus to provide another layer of protection against inhaled pathogens as a first line of defense for the immune system (Fahy et al., 2010, Lillehoj, et al., 2002).

The mucins that are secreted from secretory cells are a mixture of large glycoproteins that form polymers and bind water to keep a thin consistency. Goblet cell

secretion is primarily the mucin MUC5AC while MUC5B is secreted from different secretory cells in the airway (Fahy et al., 2010). Hypersecretion of mucus proteins due to cell hyperplasia increases the protein content of the mucus layer which leads to, among other things, viscous and sticky mucus that is much harder to clear from the airway. Mucus plugs in the lower airways are a major cause of mortality in fatal cases of acute asthma exacerbation and are related both to overproduction of mucus and dysfunctional clearance (Houston et al., 1953).

SMAD signaling

The mothers against decapentaplegic homologs (SMAD family) are the proteins that carry out signal transduction for the TGF- β and BMP receptor pathways and are critical for airway cell differentiation. Signaling of the SMAD pathway has been shown to be expressed at low levels in basal cells and goblet cells but highly active in other types of differentiated airway cells including ciliated and club cells and increased with general differentiation and maturity (Mou et al., 2016, Feldman et al., 2019). While there are other signaling pathways known to increase goblet cell production such as Notch, these function upstream of SMAD and have variable effects on goblet cell populations (McCauley et al., 2015). Therefore, SMAD signaling may be the main determinant of goblet vs nonmucous cell fate in the airway.

SMAD signaling involves multiple structurally similar proteins in regulatory, inhibitory, and cofactor roles (Figure 2). After ligands bind to the cell surface receptors, groups of regulatory SMADs are phosphorylated which exposes their nuclear localization

sequence and promotes association with the co-SMAD. All SMAD signaling requires the co-SMAD, SMAD4, for function and loss of SMAD4 abolishes SMAD signaling in the cell. After association, the SMAD complex travels to the nucleus and binds to target genes to modulate transcription (Heldin et al., 1997).

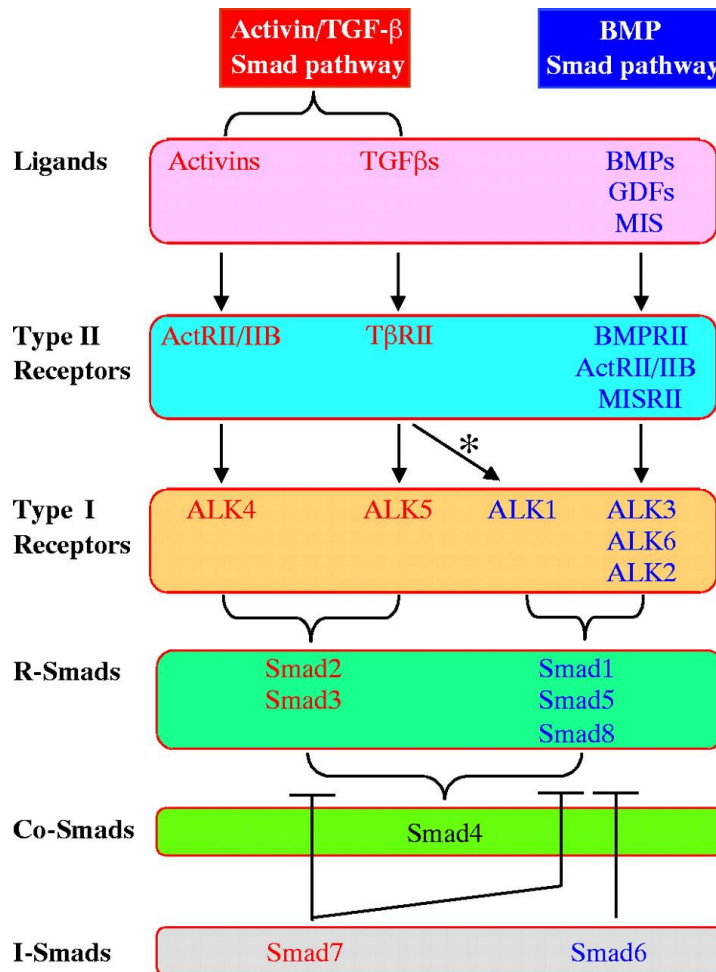


Figure 2. Overview of SMAD signal pathway. SMAD proteins 1-8 are structurally similar but involved in different roles in the pathway. They are stimulated by the ligands TGF-β (Transforming growth factor β) or BMP (Bone morphogenic protein) attaching to cell surface receptors. When triggered, regulatory SMADs (R-SMADs) are phosphorylated by the receptors and begin the signal cascade before binding the co-SMAD and their target genes. Inhibitory SMADs (I-SMADs) act on multiple different areas of the pathway. (Moustakas et al., 2001)

OBJECTIVES

The objective of this study was to determine the role of SMAD signaling in goblet cell fate determination in the airway utilizing a knockout SMAD4 cell line. Generating a cell line with knockout SMAD4 abolishes all SMAD signal pathway activity in the cell. We hypothesized that SMAD signaling is required for differentiation of ciliated and club cells. Therefore, lack of SMAD signaling would lead to only differentiation of goblet cells from basal cells. SMAD signaling level would then be the main factor in airway cell fate.

METHODS

Human airway cell isolation

As described in Mou et. al, 2016, cells used in this experiment were initially isolated from discarded human adult lung transplantation specimens collected at Massachusetts General Hospital or the New England Organ Bank. Patients granted informed consent and discarded tissue cells were collected under IRB-reviewed protocols (#2017P001479, #2013P002332, and #2016P001890). Tissues were transported and stored in PBS at 4C until use within 24 hours. The trachea and mainstem bronchi were isolated and cleared of blood cells and connective tissues. Dissected airways were cut into smaller pieces and incubated for 24 hours in DMEM/F12 medium supplemented with 1mg/mL pronase, 0.5mg/mL DNase, 5M ROCK inhibitor y27632, and 1x Penicillin-Streptomycin. After 24 hours a cell scraper was used to remove the epithelial layer which was then filtered using 100µm filters. The cells were centrifuged at 100RPM for 3 minutes and washed 3 times with DMEM supplemented with 10% FBS before being plated.

Other isolated cell types such as fibroblasts, goblet cells, and ciliated cells have more difficulty attaching to tissue culture dishes which allowed them to be removed from the cell population during media changes or caused them to die without being propagated. Epithelial cells were also separated from other cell types by using FACS with antibodies for epithelial cell adhesion molecule (EpCAM). 24 hours later the epithelial cells that attached to the tissue culture plate were stained for stem cell markers. The observed combination of p63, KRT5, and lung transcription factors NKX2.1, SOX2, and FOXA2

confirmed they were airway basal cells from a human adult. Subsequent stains of P1 and later generations showed 100% positivity rate for this combination of cell markers and indicated only human adult airway basal cells were present.

Tissue culture plate preparation

Human adult basal cells were cultured on standard tissue culture treated plates. The plates require additional pre-coating with 804G-conditioned medium supplemented with laminin to attach basal cells. To produce this conditioned media, 804G cells were grown until confluence in RPMI with 10% FBS and 1:100 penicillin/streptomycin. Culture medium was then added to cover the cells, becoming enriched with matrix proteins released by the 804G cells. The media was collected, passed through 0.4 μ m filters, and saved for future use as 804G-conditioned medium. Conditioned medium was added to cover the culturable area of the tissue culture plate. Plates were then incubated for 2 to 24 hours at 37C before removal of the 804G conditioned medium, washing with PBS, and seeding of the basal cells. Tissue culture plates used with mouse basal cells were left uncoated.

Human airway basal cell tissue culture

Airway Epithelial Basal Medium was used to culture cells using the recipe as described in Levardon et. al, 2018. Small Airway Epithelial Cell Medium (Table 2) was purchased and supplemented with ROCKi (5-10mM), A-83-01 (1mM) and CHIR99021

(0.5mM). Medium was changed daily during the culture period until the cells reached 90% confluence. Cells were passed using trypsin dissociation which was quenched with RPMI. Detached cells were centrifuged at 100rpm for 3 minutes, resuspended in airway epithelial basal medium, and seeded in new plates at a 1:10 ratio.

Mouse airway basal cell isolation and culture

SMAD4 f/f mice (Stock No:017462, Jax Lab) were used. After sacrificing the mice with CO₂ according to established protocols and regulations, the trachea was bluntly isolated and cut at the bifurcation of the mainstem bronchi. The trachea was then dissected away from attached connective tissue and other structures, cut longitudinally, and placed in 1mL of digestion buffer (20mL DMEM/F12 with 0.5mg/mL pronase, 0.5mg/mL DNase, 1% Pen-strep, 5 μ M ROCK-i). The tissue in digestion buffer was shaken at 150rpm at 37C for at least 2 hours to detach basal cells. After being centrifuged at 100rpm for 3 minutes and washed in RPMI with 10% FBS, cells were resuspended in airway epithelial basal cell media. Isolated cells were then plated on standard 6 well tissue culture plates and grown with airway epithelial basal cell medium.

Creation of SMAD4 -/- cell lines

Basal cells at P1-2 were used for the creation of SMAD4 -/- cell lines. Human airway basal cells were transfected with a Crispr-cas9 system consisting of pHIV-H2B-eGFP, human SMAD4 crRNA, and synthetic tracrRNA (Table 2). During transfection,

the tracrRNA binds the Cas9 nuclease (Jinek et al., 2012) while the crRNA targets the nuclease activity to exon 4 of the SMAD4 sequence. Together they work as the guide RNA, causing double stranded breaks and allowing insertion of the Human Histone H2B / eGFP plasmid. The addition of extra genetic material in the middle of the SMAD4 sequence disrupts normal transcription and leaves the gene nonfunctional. Cells with successful insertion express H2B tagged with GFP, allowing them to be separated from non-transfected cells. After 2 days of infection, cells were sorted to select for successfully transfected GFP positive cells. Single GFP positive cells were sorted into each well of a 96 well plate to allow for expansion of individual cells. After expansion for 14 days, successful clones were dissociated with trypsin and transferred to 6-well tissue culture plates for expansion (Figure 3). Western blot was used to confirm specific deletion of SMAD4 without change to other SMAD pathway components (Figure 4). Cells without successful transfection were used as control cell lines.

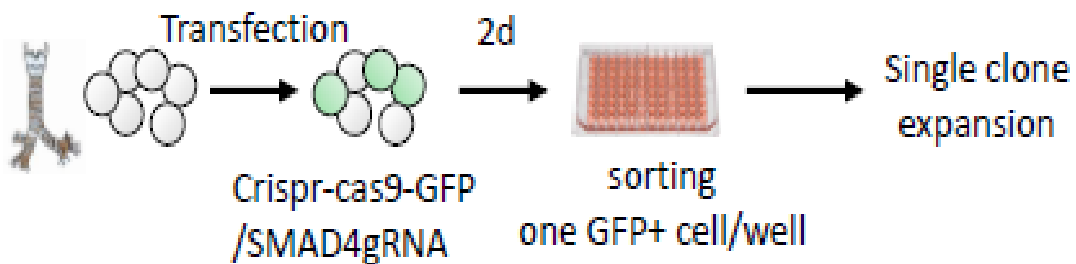


Figure 3. Overview of human SMAD4 -/- basal cell line generation. (Zhang et al., 2020)

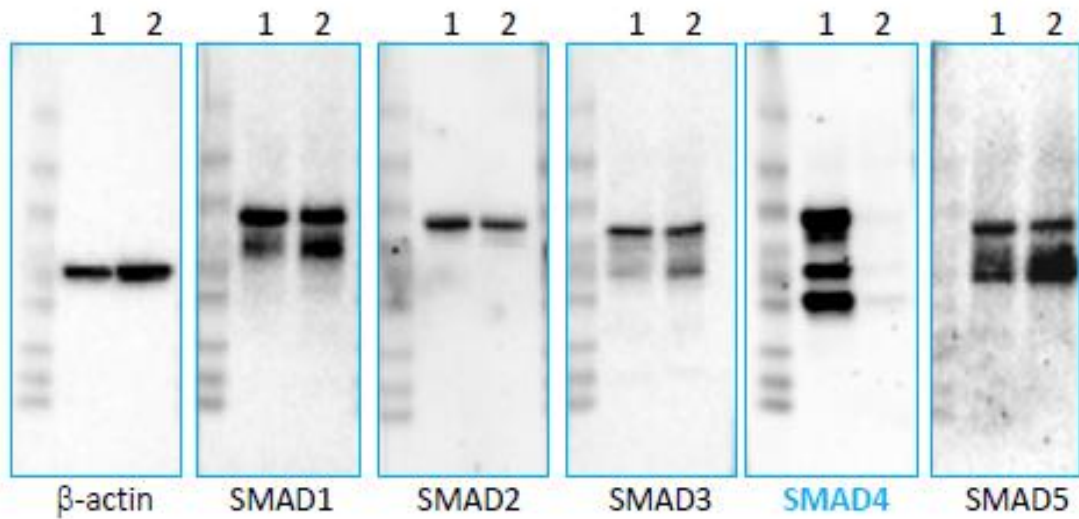


Figure 4. Western blot confirming SMAD4 ablation in human airway basal cells. SMAD proteins 1-5 are structurally similar but have different roles in the pathway. β -actin is used as a loading control. Lane 1 is the control cell line and lane 2 is the SMAD4 $-/-$ cell line. Lack of a band in lane 2 of the SMAD4 staining indicates no gene expression from a successful ablation. (Zhang et al., 2020)

Mouse SMAD4 knockout tracheal basal cell lines were generated using a similar method. SMAD4 expressing mouse tracheal basal cell lines were infected with AAV-Cre-GFP virus using the CMV promoter (Table 2) for 2 days. GFP positive cells resulting from successful knockouts were sorted and expanded as with human cells (Figure 5). SMAD4 deletion was confirmed with western blot to ensure no other SMAD pathway components were ablated (Figure 6).

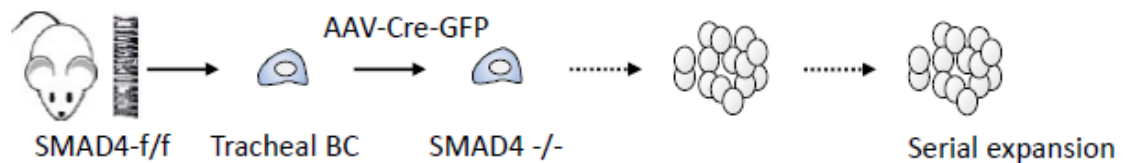


Figure 5. Overview of mouse SMAD4 $-/-$ basal cell line generation

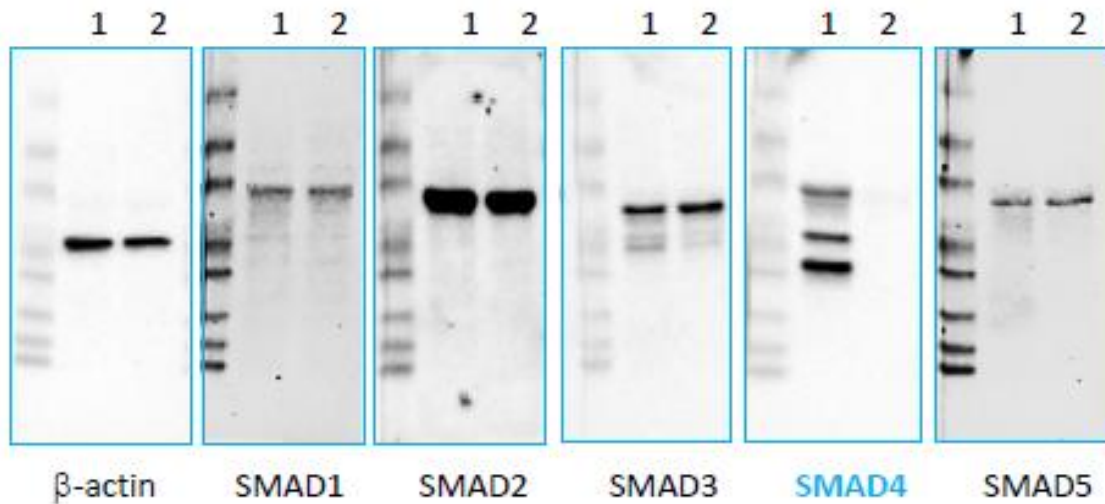


Figure 6. Western blot confirming SMAD4 ablation in mouse tracheal basal cells. β -actin is used as a loading control. Lane 1 is the control cell line and lane 2 is the SMAD4 $-/-$ cell line. Lack of a band in lane 2 of the SMAD4 staining indicates no gene expression from a successful ablation. (Zhang et al., 2020)

Air-liquid interface mucociliary differentiation of basal cells

To begin mucociliary differentiation, cells are trypsinized and moved from tissue culture plates to 0.4 μ m Transwell membranes (Levardon et. al, 2018). For the first 24 hours, complete basal cell medium was used in the upper and lower chambers. After 24 hours, any unattached cells were removed when replacing medium with complete air-liquid interface (ALI) medium. On day 3 and subsequent days, ALI medium was added only to the bottom chamber. Air exposure in the apical chamber mimics the environment in the lungs and induces mucociliary differentiation (Figure 7). Differentiation was completed over the 14-16-day culture period. Culture was extended to 4-6 weeks when required by the experiment. Murine lungs do not develop goblet cells in the same numbers as the normal human lung without stimulation of their immune system (Blyth et al., 1998, McCauley et al., 2015). Therefore IL-13 was added to the complete ALI medium for 4 days for mouse basal cell cultures to develop all cell types of interest.

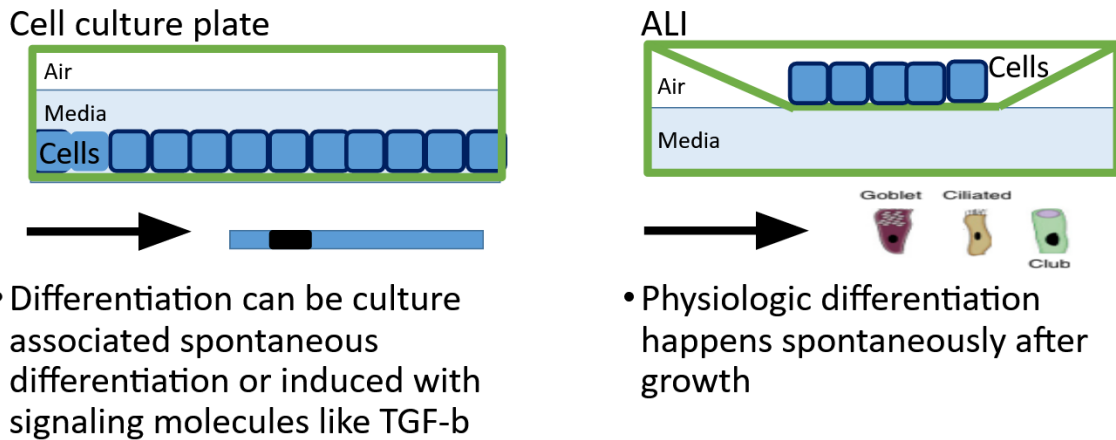


Figure 7. Description of differences between cell culture plate and ALI culture models. Cell culture models using a flat plate leave the cells submerged in media. Differentiation of airway basal cells in this model leads to flat, nonspecifically differentiated cells that are not capable of replicating. In an ALI model, exposure to air leads to physiologic differentiation with terminally differentiated and transitional cell types seen in a normal lung.

Cell transfection and culture to stimulate exogenous c-Myb expression

Ciliated cell generation was noted to be suppressed in SMAD4 knockout cell cultures. To examine the role of SMAD signaling in ciliated cell generation, exogenous c-Myb was expressed to determine if it could rescue ciliated cell generation. A SMAD4 knockout ALI was seeded and the cells were infected with a 3rd-generation doxycycline-inducible c-Myb lentivirus system with a multiplicity of infection of 10:1 (pINDUCER21-myb, Addgene #51305) for 2 days. Using the 5'LTR promoter, the lentivirus system incorporated the plasmid into a randomly located active gene. After the infection, 1 μ g/mL doxycycline was added to the complete ALI culture medium for 14 days (Figure 8).

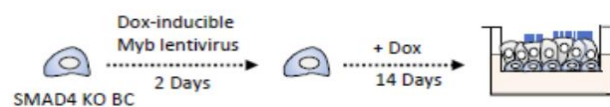


Figure 8. Outline of c-Myb lentivirus infection. (Zhang et al., 2020)

Cell fixation, immunofluorescence, and analysis

Cells were fixed for staining in 4% paraformaldehyde for 10 minutes at room temperature. After fixation, membranes were washed in PBS and permeabilized with 0.2% Triton X-100. Fixed membranes were used for wholemount staining with primary and secondary antibodies (Table 1). Phospho-SMAD staining is performed with Tyramide Signal Amplification (TSA) using a previously published protocol (Mou et al, 2016). Goblet cell differentiation was quantified after staining using ImageJ Software. The percentage of mucus-positive area out of the imaged area is scored as previously described in Feldman et al., 2019. MUC5AC images were all taken using the same exposure times and intensity adjustment parameters. Images were converted to grayscale by using an 8-bit format and the threshold was chosen to cause low-value pixels to turn red. The measured area for each image was termed the goblet cell index.

Slides are captured with an Olympus Fluoview FV10i Confocal Microscope or a Nikon A1 Confocal Laser microscope. Stained cells on culture dishes and Transwell membranes are captured with the Olympus IX81 inverted fluorescence microscope. Quantification of the images is performed with a 20x or 60x objective. 5 random fields of view are counted, and the average and standard deviation are calculated. Thicker, differentiated cell cultures are captured using multiple focal planes that are combined using MicroSuite FIVE (Olympus Soft Imaging Solutions) and Extended Focal Imaging (EFI) module to create one image.

Statistical analysis

Means with standard deviations of all measurements are shown unless otherwise described. $N \geq 3$ is used in all experiments. The Student two-tailed T-test is used to calculate any statistical differences between samples. P-values below 0.05 are reported as significant (** $p \leq 0.0001$, ** $p \leq 0.001$, * $p \leq 0.01$).

Table 1. Primary antibody list

| <i>Antibody</i> | <i>Source</i> |
|--------------------|---|
| P63 | Genetex, Rabbit monoclonal, GTX102425 |
| P63 | Abcam, Mouse monoclonal [clone 4A4], ab735 |
| Acetylated tubulin | Sigma, Mouse monoclonal [clone 6-11B-1], T7451 |
| CCSP | Sigma, Rabbit polyclonal, HPA031828-100UL |
| c-Myb | Santa Cruz Biotechnology, Rabbit polyclonal, sc-517 |
| MUC5AC | Thermo Scientific, Mouse monoclonal [clone 45M1], MA1-38223 |
| KRT5 | Abcam, Rabbit polyclonal, ab53121 |
| NKX2.1 | Abcam, Rabbit polyclonal, ab76013 or ab72876 |
| FOXA2 | Abcam, Rabbit polyclonal, ab23630 |
| SOX2 | R&D, goat polyclonal, AF2018 |
| Ki67 | eBioscience, Rat monoclonal, 14-5698-82 |

Table 2. Other products used

| <i>Description</i> | <i>Source</i> |
|--------------------------------|---|
| Small Airway Epithelial Medium | Lonza, CC-3118 or Promocell medium, C-21170 |
| pHIV-H2B-eGFP | Plasmid #91776, Addgene |
| Human SMAD4 crRNA | Dharmacon, CM-003902-01-0005 |

| | |
|--------------------------|---------------------------------|
| Synthetic tracrRNA | Dharmacon, U-002005-05 |
| SMAD4 f/f mice | Stock No:017462, Jax Lab |
| AAV-Cre-GFP virus | Vector Biolabs, 7018 |
| Pneumacult-ALI medium | StemCell Technology, Cat. 05001 |
| Y-27632 (ROCK Inhibitor) | Tocris, 1254 |
| A-83-01 (TGFb inhibitor) | Tocris, 2939 |
| CHIR99021 (WNT agonist) | Tocris, 4423 |
| Recombinant human IL-13 | Peprtech, 200-13 |
| Recombinant mouse IL-13 | Peprtech, 210-13 |

RESULTS

Previous work done with small molecule SMAD inhibition demonstrated that it allowed basal cells to retain their stemness over many passages (Mou et al., 2016, Feldman et al., 2019). SMAD signaling is a component in both the TGF and BMP signaling pathways which are critical for cell growth. As all SMAD signaling requires binding of the SMAD4 cofactor to the effector SMAD components, deletion of this ‘coSMAD’ causes complete ablation of the pathway. This genetic deletion replaced small molecule inhibitors and confirmed the results of previous experiments while removing the possibility of off target effects.

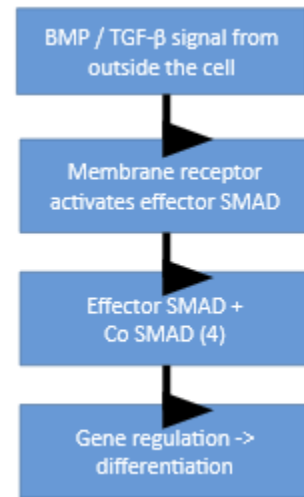


Figure 9. Brief overview of SMAD pathway.

Loss of SMAD signaling leads to basal cell cultures that maintain stemness

Data from the SMAD4 knockout basal cells supports the previous work done with small molecule inhibitors (Mou et al., 2016). Airway basal cells were cultured in medium containing A8301(TGF/SMAD antagonist) to prevent differentiation or TGFβ to stimulate it (Figure 10). The knockout cell line did not require inhibitor molecules to maintain stem cell characteristics while the control cell line did. Morphology was compared between the SMAD4 knockout cells and a control cell line. Undifferentiated basal cells retained a homogenous ‘cobble-stone’ like appearance and continued proliferating. Nonspecifically differentiated cells visibly flatten and stop proliferating.

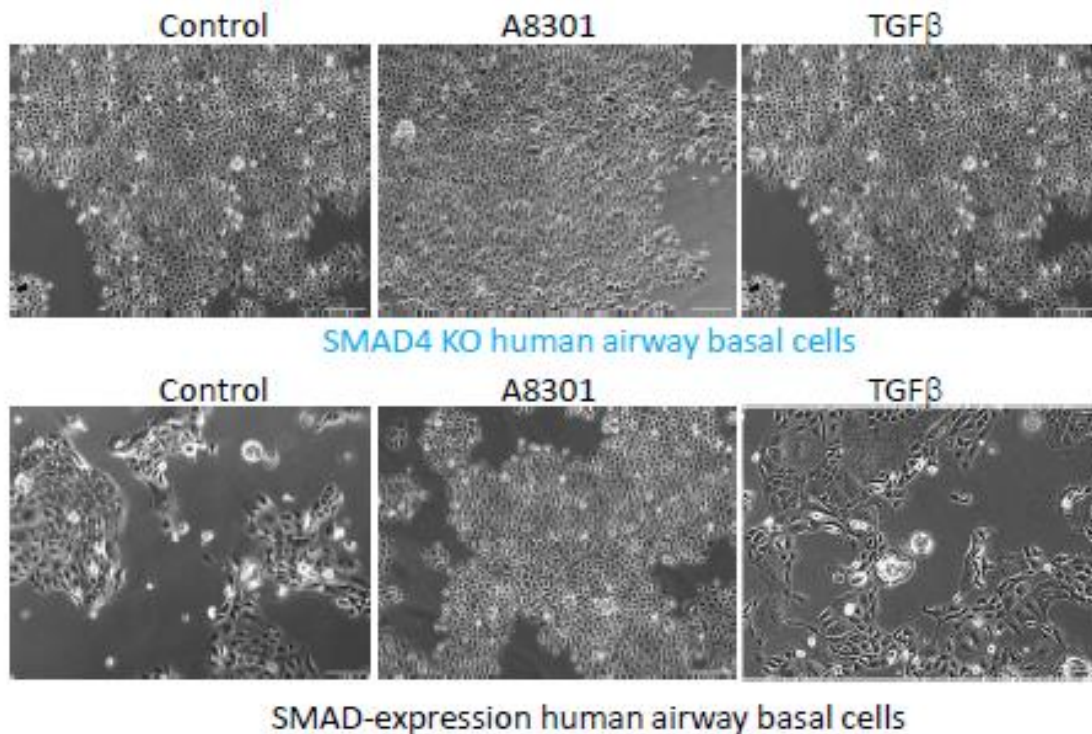


Figure 10. Comparison of basal cell morphology pre and post differentiation. SMAD4 knockout human airway basal cells are compared to a control cell line that expresses SMAD4. Cells are cultured in $1\mu\text{M}$ A8301 (a SMAD antagonist) or 10ng/mL TGF β (a signal to begin differentiation). Basal cells retain homogenous, ‘cobble-stone’ appearance while differentiated cells (bottom row, rightmost image) have a flattened appearance. Cells lacking SMAD are refractory to differentiation and only SMAD expressing cells that are not treated with the SMAD antagonist are able to differentiate. Scale bar: $100\ \mu\text{m}$. (Zhang et al., 2020)

The data supported the hypothesis that SMAD inhibition maintained stem cell characteristics of the cultures. SMAD4 knockout human airway basal cells retained the initial basal cell morphology even when stimulated to differentiate through media containing TGF β (Figure 10). It was similar to the morphology in the culture containing A8301, a SMAD antagonist. The stimulated SMAD4 knockout cells also continued to express biomarkers characteristic of baseline airway basal cells, the airway lineage markers FOXA2, SOX2, NKX2.1, and basal cell markers CK5 and P63 (Figure 11). This

suggests there is no change to other areas of the basal cell growth or maturation.

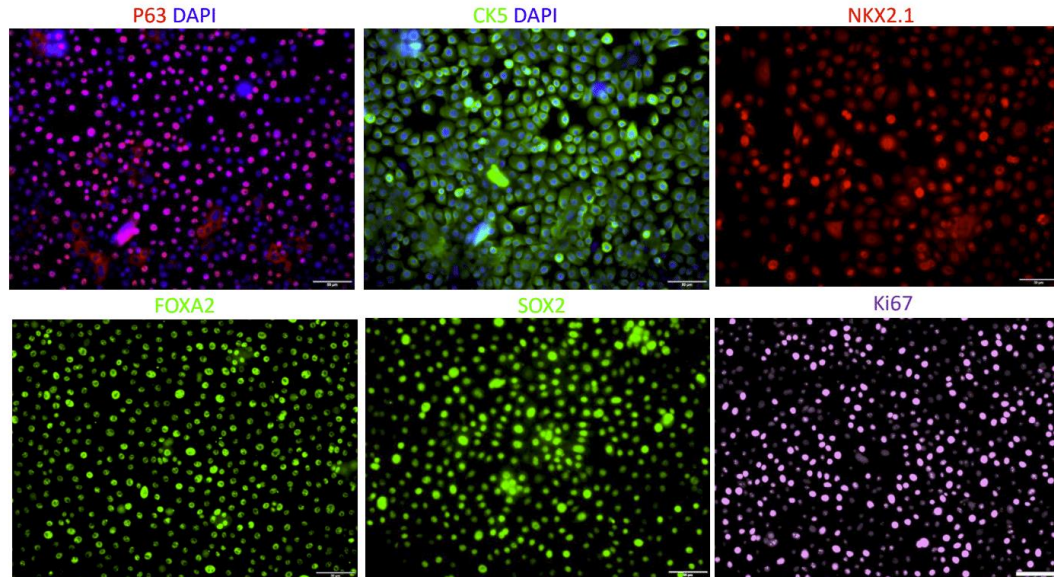


Figure 11. Immunofluorescent staining for basal cell biomarkers in SMAD4 knockout human airway basal cells. Post TGF β stimulation basal cells have retained their stem cell characteristics. The combination of these markers is characteristic of basal cells. DAPI stain indicates cell nuclei. Scale bar: 50 μ m. (Zhang et al., 2020)

In contrast to the SMAD4 knockout cell line, the SMAD4 expressing cell line required SMAD inhibition by A8301 to avoid nonspecific terminal differentiation (Figure 10).

Similar results were expected between the control and SMAD4 knockout mouse tracheal basal cell lines. This was supported by the results obtained (Figure 12). Knockout cells retained their initial morphology and did not display CK8, a biomarker indicating differentiation. SMAD4 expressing cells displayed the opposite.

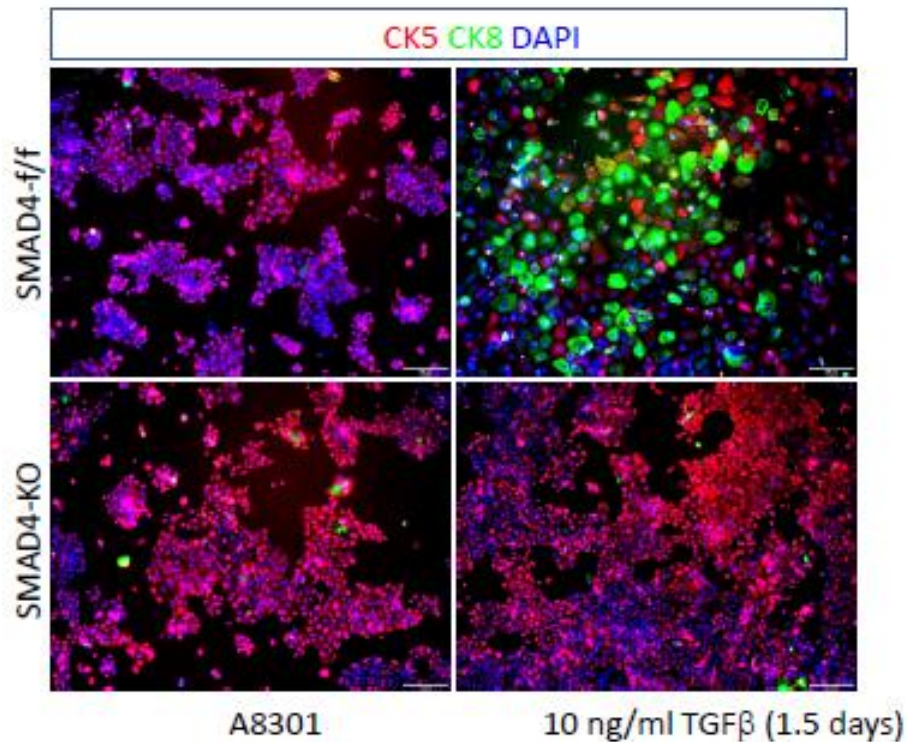


Figure 12. Immunofluorescent staining for basal cell biomarkers in mouse tracheal basal cells. Cultured in a standard tissue culture plate. The combination of these markers is characteristic of basal cells. DAPI indicates cell nuclei. Scale bar: 100 μ m. (Zhang et al., 2020)

Inhibition of SMAD signaling either by inhibitors or genetic knockout allowed basal cells to continue proliferating in culture without terminally differentiating and retain stem cell characteristics through passages.

SMAD signaling level correlates with the differentiated cell types that are generated

Goblet cells, ciliated cells, and club cells have been noted to have different levels of SMAD signaling (Mou et al., 2016). SMAD signaling has been reported to increase in ciliated and club cells relative to basal or goblet cells. We hypothesized that ciliated and club cells are high SMAD cell types which require SMAD signaling while goblet cells

are a low SMAD cell type that does not. When human SMAD4 expressing and knockout cells were plated on the ALI model to mimic normal differentiation in the lung environment, there were no ciliated cells noted (Figure 13). When the culture period was extended to 5 weeks there was no change in ciliated cell generation. This suggested that cells were not capable of ciliated cell generation without SMAD signaling rather than a delay in differentiation (not shown).

Club cells were noted at day 3 in SMAD4 expressing cell culture and populations increased daily (Figure 14). Unexpectedly for a higher SMAD signaling cell type, small populations of club cells were seen in the SMAD4 knockout culture. It is possible that club cells are heterogeneous and not all require SMAD signaling. It is also possible but less likely that the H2B/GFP insertion was off target or a gain of function mutation allowed these cells retain some level of SMAD signaling. Western blot confirmation indicated the genetic knockout that began the cell line was successful.

Goblet cell generation was not affected by SMAD deletion. In both cell lines, goblet cells are noted starting at day 3 (Figure 15). This is consistent with goblet cells as a low SMAD cell type that is not affected by loss of SMAD signaling (Feldman et al., 2019).

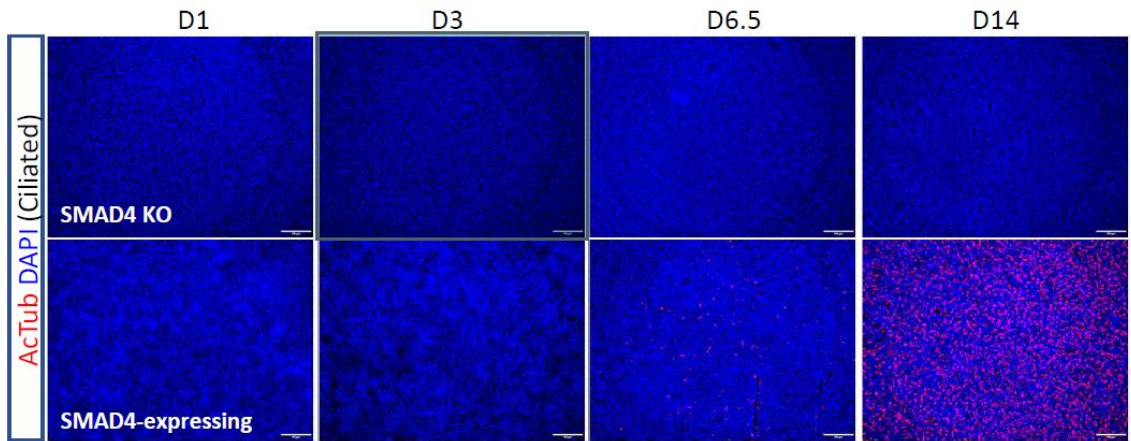


Figure 13. Immunofluorescent staining for ciliated cell biomarker in an ALI culture of human airway basal cell culture. Acetylated tubulin (AcTub) indicates ciliated cells. DAPI is used to stain cell nuclei. No ciliated cells were seen in the SMAD4 KO cells from 0-4 weeks. Ciliated cells were seen in SMAD4 expressing cells at 6.5 days. Scale bar: 100 μ m. (Zhang et al., 2020)

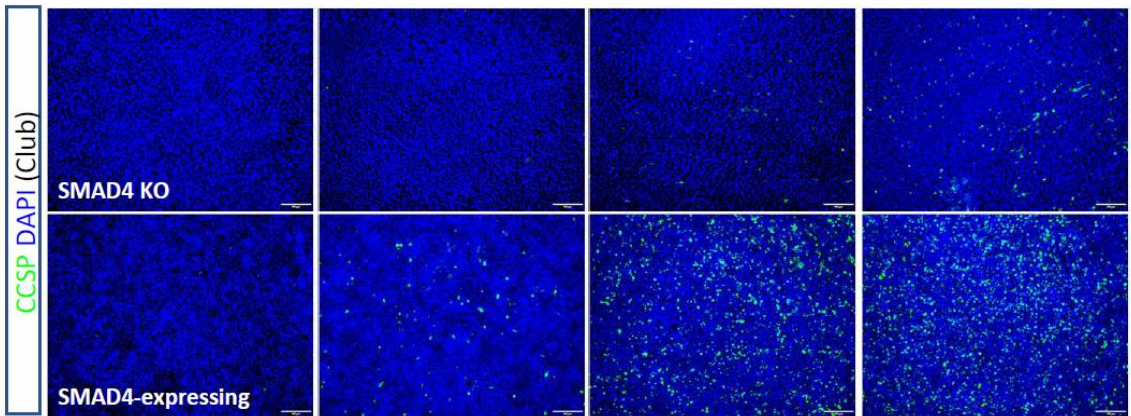


Figure 14. Immunofluorescent staining for club cell biomarker in an ALI culture of human airway basal cell culture. Club cell secretory protein (CCSP) indicates club cells. DAPI is used to stain cell nuclei. Small populations noted in SMAD4 KO cells while normal amounts are seen in SMAD4 expressing cells. Days 1, 3, 6.5, and 14 respectively. Scale bar: 100 μ m. (Zhang et al., 2020)

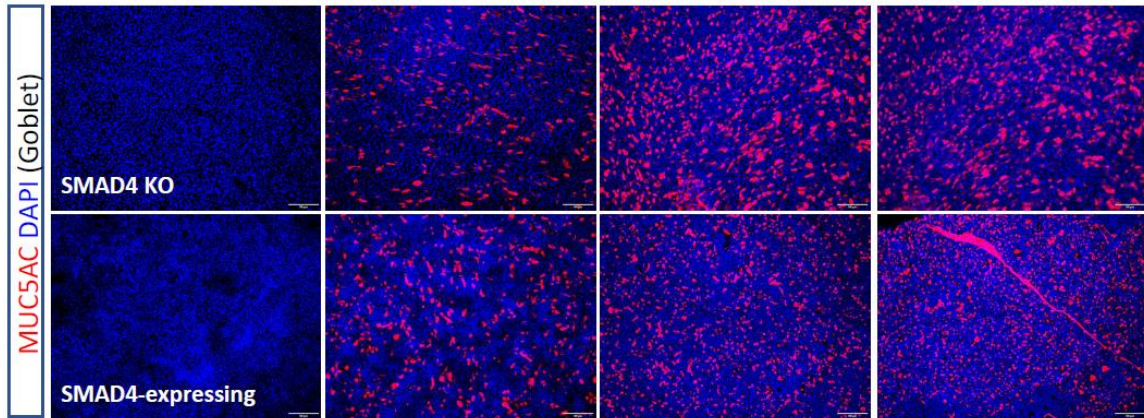


Figure 15. Immunofluorescent staining for goblet cell biomarker in an ALI culture of human airway basal cell culture. Mucin 5AC (MUC5AC) indicates goblet cells in this image as it is the primary mucin produced by this cell type (Fahy et al., 2010). MUC5AC is also produced by a subset of other secretory cells (Okuda et al., 2019) but is primarily showing goblet cells in this image. DAPI is used to stain cell nuclei. Days 1, 3, 6.5, and 14 respectively. Scale bar: 100 μ m. (Zhang et al., 2020)

The results of this experiment were replicated in cultures of human airway basal cells isolated from separate patients (Figure 16).

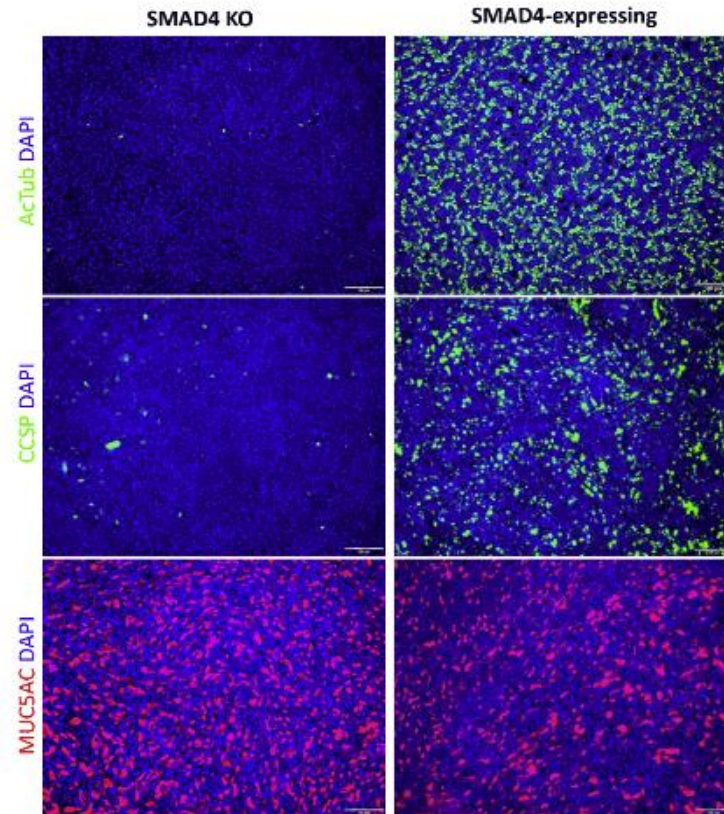


Figure 16. Immunofluorescent staining for differentiated cell biomarkers in an ALI culture of human airway basal cells. Mucin 5AC (MUC5AC) indicates secretory cells (Okuda et al., 2019), used here to indicate goblet cells as it is the primary mucin produced by goblet cells (Fahy et al., 2010) and is produced by only a subset of club cells. Acetylated tubulin (AcTub) indicates ciliated cells and club cell secretory protein (CCSP) indicates club cells. DAPI is used to stain cell nuclei. The culture is imaged at day 14. Scale bar: 100 μ m. (Zhang et al., 2020)

In mice, results were expected to be identical to results in humans. We tested the effect of SMAD4 deletion in mouse tracheal basal cells using ALI culture (Figure 17). Like human basal cells, no ciliated cells differentiated in the SMAD4 deletion cell line. No club cells were noted in the mouse tracheal basal cell culture. Mice do not generally produce goblet cells at baseline, so IL-13 was added to the media for the final 4 days of culture to stimulate goblet cells (Blyth et al., 1998, Kuperman et al., 2002, McCauley et al., 2015). Unexpectedly, no goblet cells were produced in the SMAD4 knockout culture when stimulated with IL-13. In contrast, goblet cells were generated in the SMAD4

expressing cells stimulated with IL-13. Together these data suggest the three major populations of terminally differentiated cells were not present in the mouse cell cultures lacking SMAD4. Although initial deletion was confirmed by western blot, additional staining that confirms no SMAD signaling was present in the cultures would support the current data.

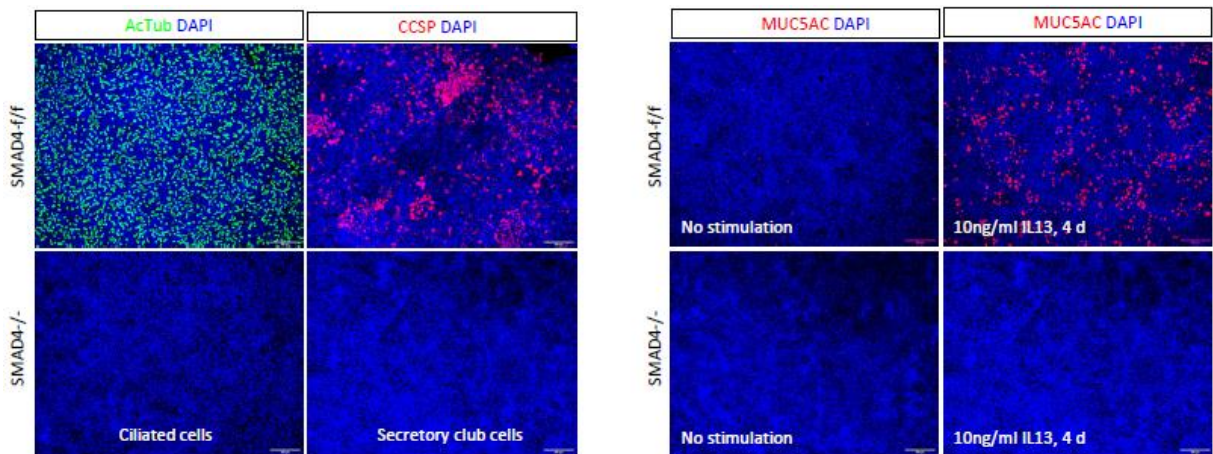


Figure 17. Immunofluorescent staining for differentiated cell biomarkers in an ALI culture of mouse tracheal basal cells. Mucin 5AC (MUC5AC) indicates goblet cells in this image, Acetylated tubulin (AcTub) indicates ciliated cells, and club cell secretory protein (CCSP) indicates club cells. DAPI is used to stain cell nuclei. The culture is imaged at day 14. Mice require IL-13 stimulation to produce goblet cells and 10ng/mL IL-13 was added to the media for the final 4 days. Scale bar: 100µm. (Zhang et al., 2020)

To understand what types of cells were present on day 14 in the SMAD4 knockout ALI and confirm there was no other injury to the cells that prevented them from differentiating, the cells were stained for a variety of biomarkers. It was determined that the cells displayed P63, CK5, and CK8 (Figure 18) which identified them as early committed precursor cells rather than basal cells.

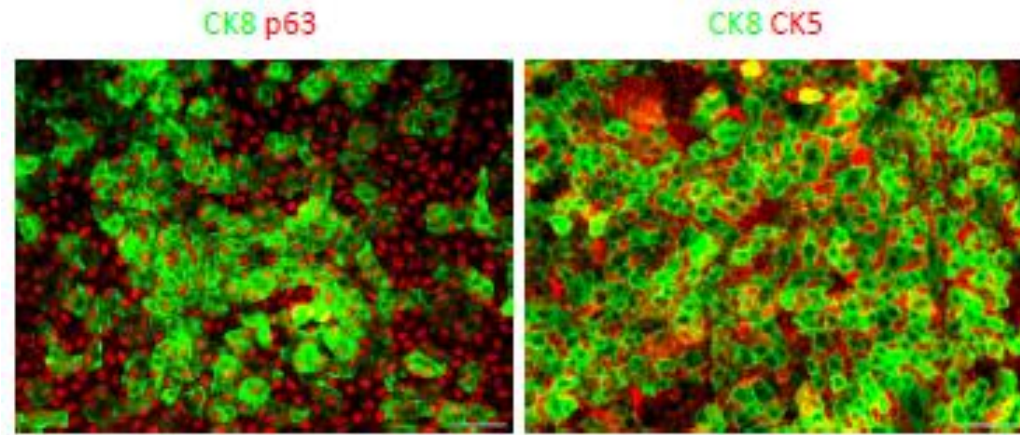


Figure 18. Immunofluorescent staining for basal cell biomarkers in SMAD4 knockout mouse tracheal basal cells. The observed combination of CK8, p63, and CK5 identify these cells as committed precursor cells that have arisen from the initial basal cells in an ALI culture. Scale bar: 50 μ m. (Zhang et al., 2020)

Ciliated cell generation requires SMAD signaling

Generation of ciliated cells has been found to rely heavily on expression of the transcription factor c-Myb (Pan et al., 2014). While basal cells do not express c-Myb, committed ciliated precursors begin to exhibit sustained and increasing c-Myb expression (Pan et al., 2014). The transition from c-Myb negative to c-Myb positive to AcTub positive indicates the transition from basal cell to precursor to ciliated cell (Figure 19).

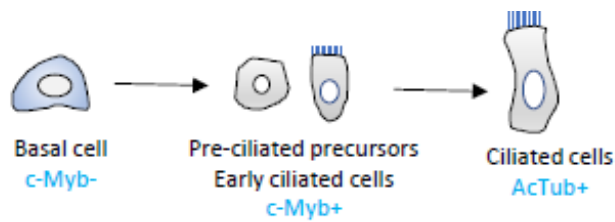


Figure 19. Simplified outline of ciliated cell differentiation and relevant protein expression. (Zhang et al., 2020)

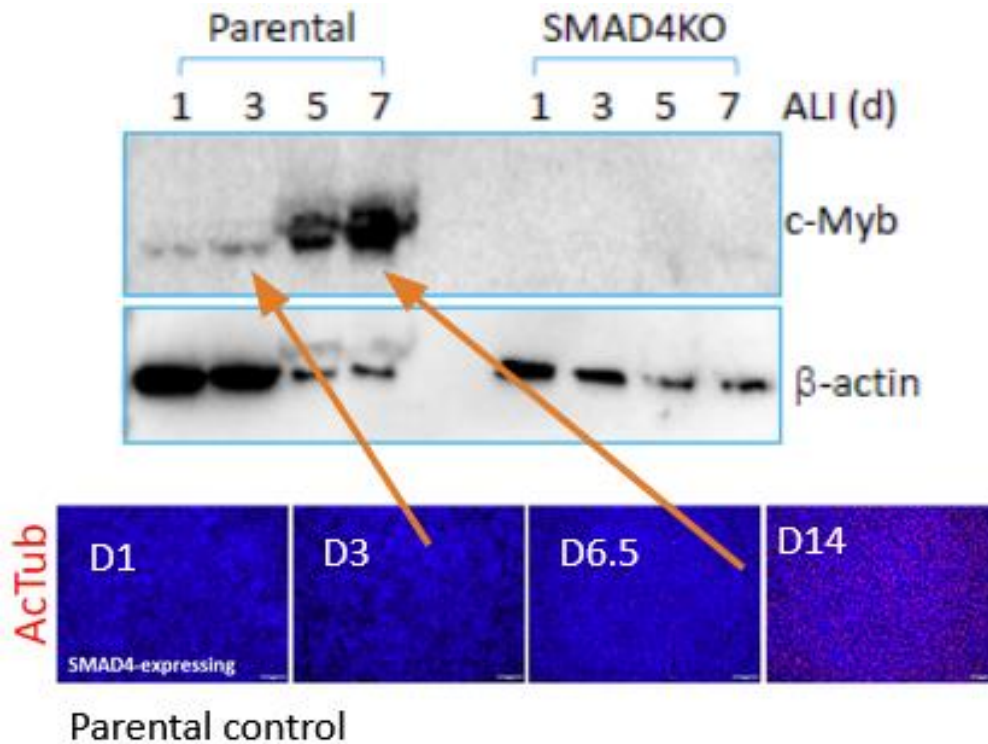


Figure 20. Western blot of c-Myb expression over time. C-myb increases over time as basal cells become committed precursors. Parental (control) and SMAD4 KO cells are contrasted. AcTub stained image for comparison of growth at each time point. No AcTub expressing cells (ciliated cells) are visible at day 3 using fluorescent staining. Some ciliated cells are visible at day 6.5 and the western blot confirms corresponding increases in c-Myb and therefore increases in ciliated precursor populations. By day 14, the large amount of c-Myb expression has translated to large numbers of developed ciliated cells. B-actin used as a loading control. (Zhang et al., 2020)

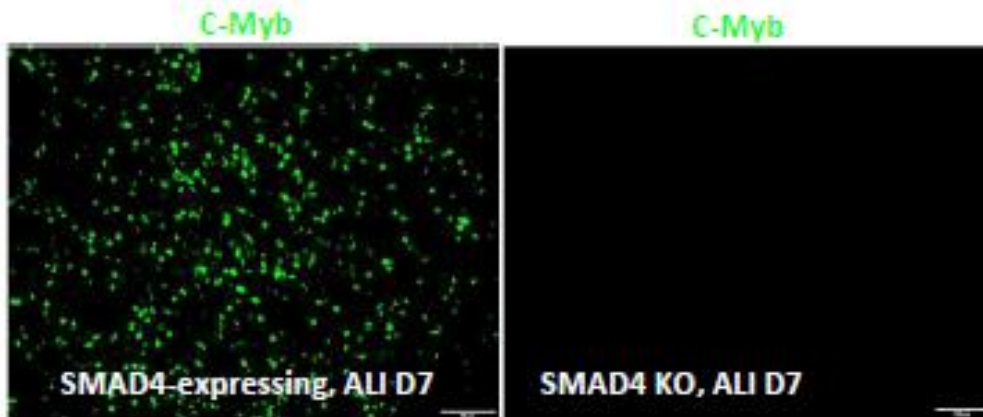


Figure 21 Immunofluorescent staining of c-Myb expression at day 7. SMAD4-expressing (control) and SMAD4 KO cells are contrasted. Scale bar: 100μm. (Zhang et al., 2020)

As expected based on the lack of ciliated cells in the SMAD4 knockout cell lines, these cells did not express c-Myb (Figure 20, figure 21). In contrast, the SMAD4 expressing control cells had increasing amounts of c-Myb expression that correlated with later ciliated cell development. Cells lacking SMAD signaling (Figure 20) also lacked c-Myb expression, suggesting SMAD signaling could be upstream of c-Myb activation. To determine if exogenous c-Myb could rescue ciliogenesis in SMAD4 knockout cell culture, cells were infected for 2 days with a doxycycline inducible c-Myb lentivirus. After stimulation with doxycycline for 14 days, cells were imaged. C-Myb expression was successfully restored in these SMAD4 knockout cells (Figure 22), however no ciliated cells were seen. Together these data suggest SMAD signaling is necessary for ciliated cell development.

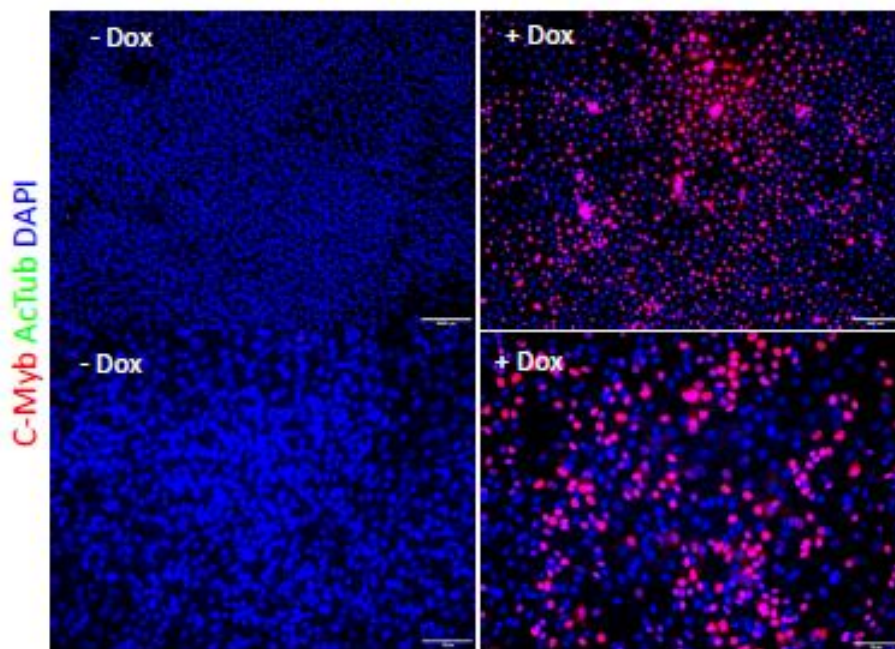


Figure 22. Immunofluorescent staining of c-Myb expression in SMAD4 KO cells after doxycycline inducible lentivirus infection and doxycycline stimulation. No c-Myb appears without doxycycline. No AcTub (ciliated cells) develop in either culture. Two views are presented. Scale bar: 100 μ m and 50 μ m. (Zhang et al., 2020)

DISCUSSION

Building on previous work, we show that SMAD signaling level is an important determinant of basal cell fate. SMAD inhibition has previously been shown to decrease doubling time and increase the number of basal cell doublings possible in cultured cell lines (Mou et al., 2016). SMAD signaling level in this pathway was also shown to increase with differentiation (Mou et al., 2016, Feldman et al., 2019). The maintenance of stemness through SMAD inhibition was confirmed with the current genetic knockout model. SMAD knockout cells were able to generate goblet cells at higher than normal levels, but not able to differentiate into normal levels of the other two cell types of interest under tested conditions (Figure 10, Figure 24). The SMAD knockout cells showed normal biomarker expression, suggesting that there were no off target effects on the cell as a result of losing this signal pathway. The changes resulting from SMAD inhibition allowed basal cells to be cultured and maintain their basal cell characteristics. Allowing basal cells to be cultured over a longer period of time instead of nonspecifically differentiating offers new opportunities for airway cell research.

The current data fits with the basal cell's characterization as a 'low SMAD' cell type (Feldman et al., 2019) (Figure 23).

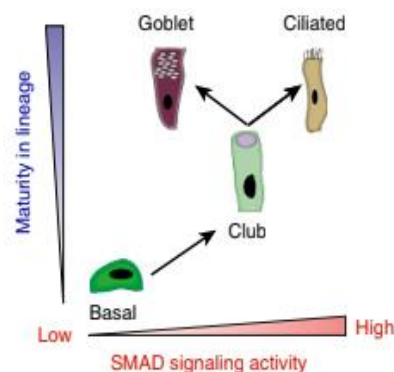


Figure 23. Previously proposed simplified airway cell differentiation model (Feldman et al., 2019)

We have shown that high SMAD signaling cell types such as ciliated cells are not generated in SMAD deletion human basal cell lines in vitro (Figure 25). Based on the normal expression of basal cell biomarkers, this suggests that SMAD signaling is a required step in the pathway of ciliated cell generation.

The club cells that act as a precursor for ciliated cells and have a correspondingly high SMAD signaling level were not seen in normal levels in the SMAD deletion basal cell lines. Although small populations of club cells were unexpectedly seen in the knockout cell line (Figure 25), when compared to a control culture the majority of expected club cells were not generated. It is possible that club cell populations are heterogeneous, and some do not rely on SMAD signals, giving rise to the small populations seen. However, the small population of club cells that developed in the SMAD deletion cells did not translate into ciliated cell generation. Even when the culture period was extended significantly there were no ciliated cells generated. This suggests that club cells that generate without SMAD signaling activity do not act as ciliated cell precursors. More data is needed to determine the fate of these club cells. It is likely that SMAD signaling activity is generally necessary for club cell generation whereas it is always necessary for ciliated cell generation.

In contrast, goblet cell generation was not affected by loss of SMAD signaling. This confirms the previous results generated with small molecule inhibitors and characterization of goblet cells as a low SMAD signaling level cell type. Compared to the control cell line there was a significant increase in the number of goblet cells generated

(Figure 24). It has been previously suggested that lack of SMAD signaling changes ciliated cell precursors to the goblet cell lineage (Feldman et al., 2019). This could be responsible for the increased generation of goblet cells in the SMAD deletion cell line.

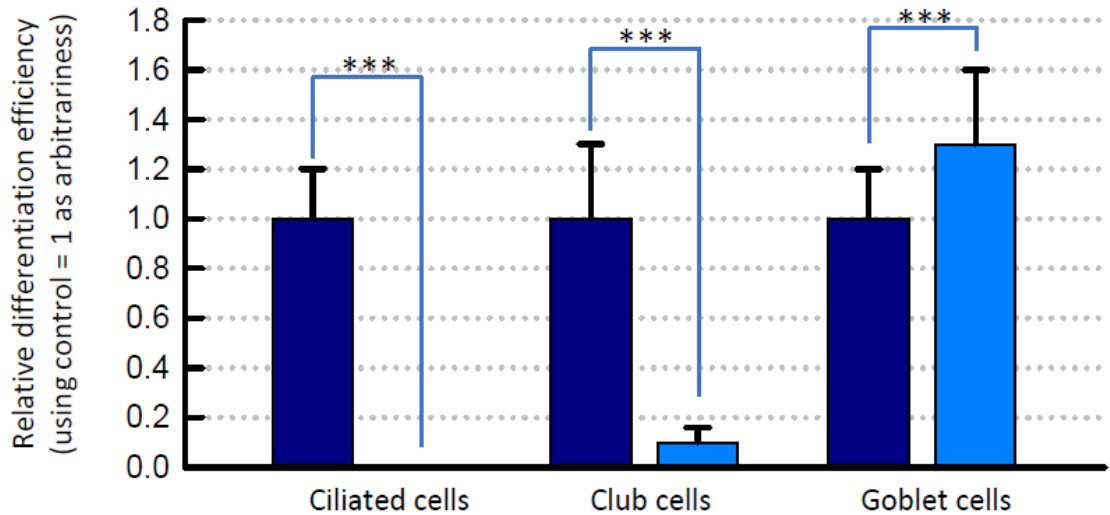


Figure 24. Relative differentiation efficiency of ciliated, club, and goblet cell generation in human control and SMAD knockout cultures. Corresponding immunofluorescence is shown in figures 13, 14, and 15. No ciliated cells are seen, a small population of club cells is seen, and more goblet cells are seen (light blue) compared to control (dark blue). (mean \pm s.d. n=3; ***p \leq 0.0001). (Zhang et al., 2020)

The observed goblet cell generation indicates different differentiation pathways for goblet cells. Goblet cell generation was seen in human SMAD knockout culture before club cell generation (Figure 14, 15). The previously proposed model for terminally differentiating airway cells (Figure 23) theorized that ciliated and goblet cells arose from club cell precursors. Our culture had a large population of goblet cells noted on day 3, but no corresponding population of club cells to act as precursors. The club cells did not appear in large numbers in the SMAD deletion cell lines at any point in culture (Figure 24). This suggests that these goblet cells did not have a club cell precursor, instead differentiating straight from basal cells. These data support the addition of a new

differentiation pathway for goblet cells to the model of airway cell differentiation (Figure 25).

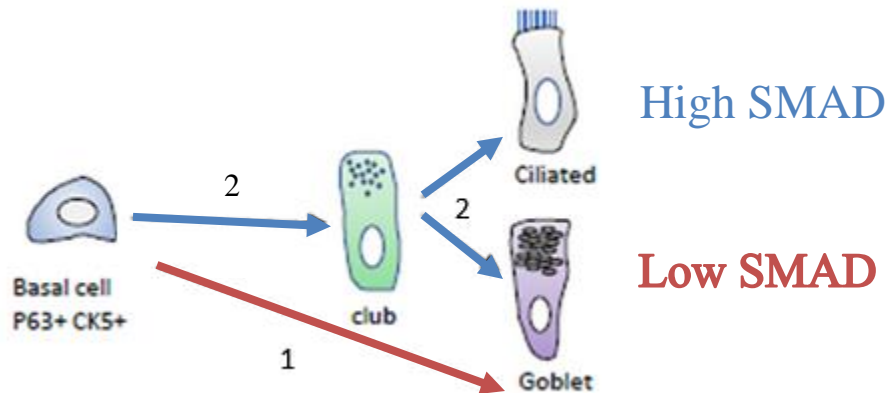


Figure 25. Newly proposed simplified model of human airway cell differentiation. Basal cells were previously thought to follow pathway 2 and differentiate from club cell precursors. Our data showing goblet cell generation before club cells are present supports adding pathway 1. (Zhang et al., 2020)

More data comparing the effects of SMAD signaling loss with other signaling pathways known to induce goblet cells would clarify the SMAD signaling pathways role in goblet cell development.

In mice, the data was not consistent with humans. No ciliated or club cells were seen, possibly supporting these cells as high SMAD signaling cell types (Figure 17). However, no goblet cells were seen after stimulation which was unexpected. As a low SMAD signaling cell type, goblet cells were expected to appear in normal amounts when there was stimulation by IL-13. The lack of terminally differentiated cells indicated significant differences between the differentiation pathways of mice and humans. Future imaging showing phospho-SMAD staining to indicate if SMAD was expressed at any level in the culture would clarify the results obtained. Immunofluorescence showed that cells were displaying biomarkers characteristic of previously reported luminal basal

precursor cells (Watson et al., 2015) rather than remaining basal cells. The production of basal precursors most likely requires other signaling processes present during physiologic differentiation rather than single ligand stimulation. This could explain our results where precursors were seen with ALI culture (Figure 18) but did not appear when the pathway was stimulated with TGF- β in a cell culture plate (Figure 12). This suggests that in the mouse model, there are not only high and low SMAD signaling cell types but SMAD signaling dependent and independent steps (Figure 26).

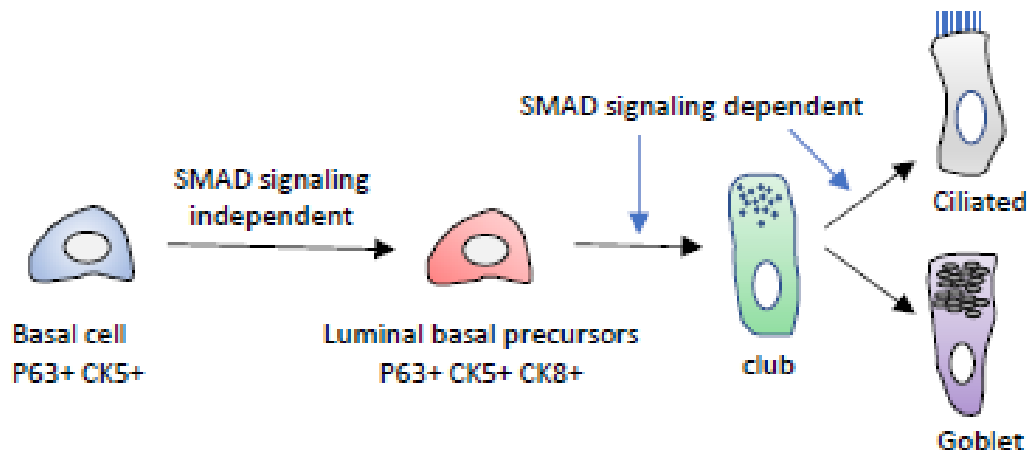


Figure 26. Proposed simplified mouse tracheal basal cell differentiation pathway. SMAD signaling independent steps are likely present in the initial part of the pathway and SMAD signaling dependent steps at the end (Zhang et al., 2020)

Ciliated cells are known to rely on c-Myb as a key early regulator of specification and differentiation (Pan et al., 2014). Pre-ciliated precursors and early ciliated cells are reliant upon c-Myb signaling activation. In the SMAD deletion cell lines where no ciliated cells are generated, it is clear that SMAD signaling is an important requirement for ciliated cell development and related to the activation of c-Myb signaling. In the control cells we detected increasing amounts of c-Myb expression that correlated with the

basal cell to ciliated precursor cell transition. However, stimulated physiologic differentiation did not lead to c-Myb expression in the SMAD4 knockout cells. This result suggests SMAD signaling activation could be the earliest event in ciliated cell development, upstream of c-Myb. Loss of SMAD likely interferes with the early development of pre-ciliated and early ciliated cell populations, preventing basal cells from committing to ciliated cell precursors. We further demonstrated that long-term sustained exogenous c-Myb expression could not rescue ciliated cell development. Although c-Myb is a necessary early regulator, it is not sufficient for ciliated cell generation without SMAD signaling.

The SMAD signal pathway has been shown to be an important regulator of airway basal cell fate. Aerosolized SMAD signaling agonists could eventually represent an effective targeted treatment for the many airway diseases that involve goblet cell hyperplasia. Loss of SMAD signals greatly increases goblet cell development at the expense of other cell types. In the future, ablating SMAD signaling from an already developed culture could show if SMAD persistence is required to maintain the balance of cell types in the lungs. SMAD signaling loss maintains the basal cell character of cell cultures, allowing for easier and more consistent use in research. SMAD signals also likely represent a key regulator of ciliated cells, suggesting the SMAD pathway may be involved in multiple fundamental functions in generating terminally differentiated cell types.

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CURRICULUM VITAE

